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Effect of Rifampin, Clofazimine, and B1912 on the Viability of *Mycobacterium leprae* in Established Mouse Footpad Infection (39276)

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Estimation of the morphological index (M.I.) of viability of *Mycobacterium leprae* in stained smears from infected skin sites is one of the criteria used for the rapid evaluation of the efficacy of leprosy chemotherapy, particularly during the early stages of treatment (1). It has been found that in a smear stained by the Ziehl-Neelsen (ZN) method, a leprosy bacillus that is uniformly and deeply stained along its full length ("solid" appearance) may be assumed to have been viable, and that on the contrary, one with a defect of staining ("nonsolid") is nonviable (2). The overall viability of a bacillary suspension is thus assessed by determining the proportion (%) of solid bacilli present. This constitutes the solid ratio (S.R.) for the smear. The M.I. is the percentage viability of bacilli derived as the mean of S.R.'s in smears from a number of sites in the skin of a leprosy patient. In view of these clinical implications, it is important to investigate the effect of potential antileprous compounds on the S.R. of *M. leprae* in experimental animals.

Rifampin (RMP) is rapidly bactericidal against *M. leprae,* both in man and in the mouse footpad infection (3-5). Several clinical trials of the efficacy of RMP treatment in lepromatous leprosy have been reported and are still in progress (3, 6, 7). Clofazimine (B663) is inhibitory to *M. leprae* growth, clinically and in mice (8-13) and is one of the second-line anti-leprous compounds in clinical use, particularly in cases of resistance to dapsone (DDS) (14, 15). B1912 is an analog of B663 which has shown suppressive activity against *M. leprae* in the mouse footpad (16, 17).

The present work was undertaken to determine the effects of RMP, B663 and B1912 on the S.R. of *M. leprae* in an established mouse foot-pad infection and to relate these findings to previously reported clinical and experimental estimates of bactericidal activity.

Materials and methods. The strains of *M. leprae* used were all derived from the tissues of previously untreated lepromatous leprosy patients, and had been maintained by serial passage in mouse footpads. They were all fully sensitive to DDS, in that they would not grow in mice receiving 0.0001% DDS in the diet. The detailed methods of mouse inoculation, assessment of bacillary growth in footpad homogenates, preparation of mice with an established footpad infection, and drug-diet preparation, have been previously described (4, 5, 16). When the bacillary number had reached 5×10^5 to 1×10^6 per footpad, the animals (ASH/CSI strain of mice) were divided into controls and groups treated with the following drugs: rifampin (RMP, Lepetit Pharmaceuticals Ltd., U.K.); and the riminophenazines clofazimine (B663, Geigy Pharmaceuticals Ltd., U.K.) and B1912 (May & Baker Ltd., U.K.). Drugs were administered in the diet either continuously (RMP, B663, and B1912) or in an intermittent regimen of 1 day in each 30 days for a period of 5 months (B663 and B1912). RMP was administered at 0.03% in the diet (equivalent to 75 mg/kg/day) and B663 and B1912 at 0.01% (25 mg/kg/day) by continuous and 0.03% by intermittent regimen.

In order to determine the effect of RMP in the absence of host immune response, mice were inoculated with 10^6 *M. leprae* and immediately immunosuppressed with anti-mouse-thymocyte globulin (ATG; 0.2 ml sc twice weekly) and 4.0 mg/kg/day hydrocortisone acetate (HC) im. A control immunologically unaltered group of mice was treated with saline only. Immunosuppressed animals were divided into a control group and a group treated with RMP, 0.03% in

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the diet, from the day of inoculation. ATG was prepared in rabbits and its activity was standardized by a skin-graft technique (18).

At intervals, the S.R. of *M. leprae* was determined in Z.N.-stained smears of homogenates of individual footpads from three mice per group. Estimations were based on the observation of 200 randomly selected bacilli from each smear; smears were randomized so that their source would be unknown to the observer. The S.R. was defined as the mean percentage of bacilli which had stained uniformly and deeply (1, 19).

Results. Continuous drug administration. In all experiments a marked exponential fall in the S.R. of *M. leprae* in control animals was noted during the first 60–80 days. The phenomenon has been previously reported and attributed to host immune response (19). The decline in S.R. in RMP-treated animals, in which both drug and host immunity could operate, was considerably more marked and continued during the whole period of observation (Fig. 1). A solid ratio of less than 5% was attained after treatment for only 20 days and treatment for 60 days was sufficient to reduce the S.R. to zero (no solids in 200 bacilli). At this stage, the S.R. in the controls was greater than 10%. The estimated half-life ($t_{1/2}$) of solid bacilli in RMP-treated animals was 6–7 days (26 days in control). In B663- and B1912-treated animals (Fig. 2) the rate of fall of the S.R. did not differ from that in the controls during the first 80 days of observation. Subsequently, the rate of change in S.R. was somewhat enhanced by drug action although a value of zero was not attained even after treatment for 350 days. The $t_{1/2}$ of solid bacilli in B663- and B1912-treated mice was 55 and 50 days, respectively (75 days in controls).

Intermittent riminophenazine administration. Intermittent administration of B663 and B1912 (Fig. 3), as with continuous administration, did not significantly affect the rate of decline in S.R. during the first 80–100 days. Subsequently, however, the rate was slightly enhanced; a value of zero was only just attained with B1912 during the period of observation. The estimated $t_{1/2}$ of solid bacilli in B663- and B1912-treated mice was 60 and 38 days, respectively (100 days in controls).

Immunosuppressed mice. There was an initial 60% loss of stainable bacilli from mice in all three groups, followed by a lag period. Then from the sixty-fifth day the total bacillary numbers in the ATG/HC-

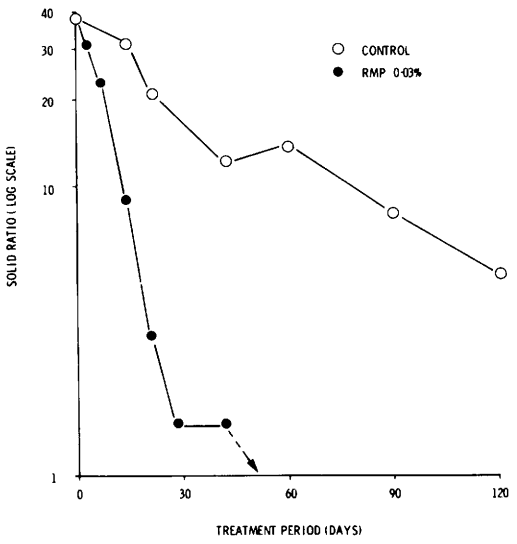

FIG. 1. The effect of continuous dietary administration of rifampin (RMP; 0.03%) on the solid ratio of *M. leprae* in an established mouse footpad infection. Values represent the mean of three replicate determinations.

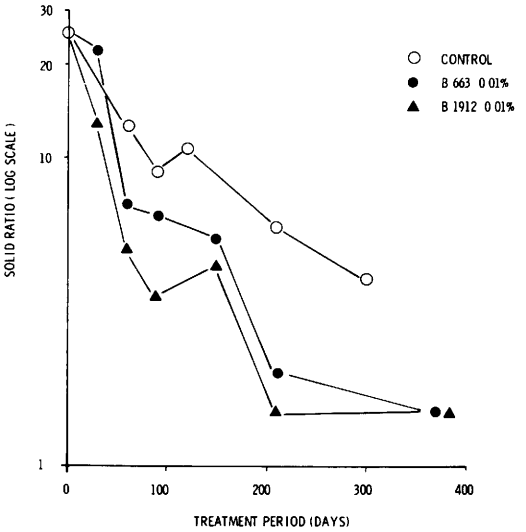

FIG. 2. The effect of continuous dietary administration of clofazimine (B663; 0.01%) and B1912 (0.01%) on the solid ratio of *M. leprae* in an established mouse footpad infection. Values represent the mean of three replicate determinations.

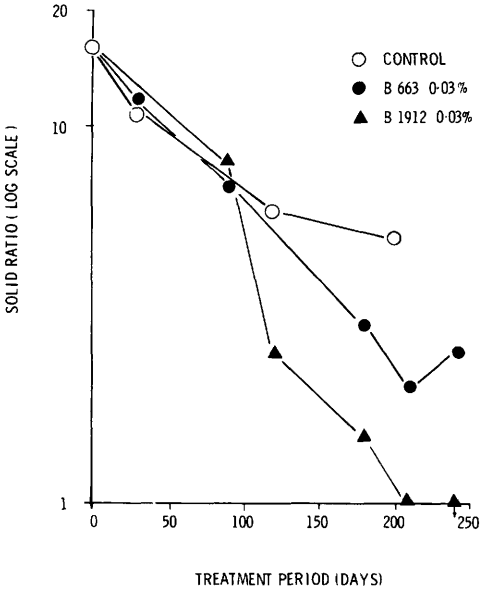

FIG. 3. The effect of intermittent dietary administration of clofazimine (B663; 0.03%) and B1912 (0.03%) on the solid ratio of *M. leprae* in an established mouse footpad infection. Drugs were administered for a 24-h period in each 30 days. Values represent the mean of three replicate determinations. Arrow (↓) indicates a solid ratio of less than one.

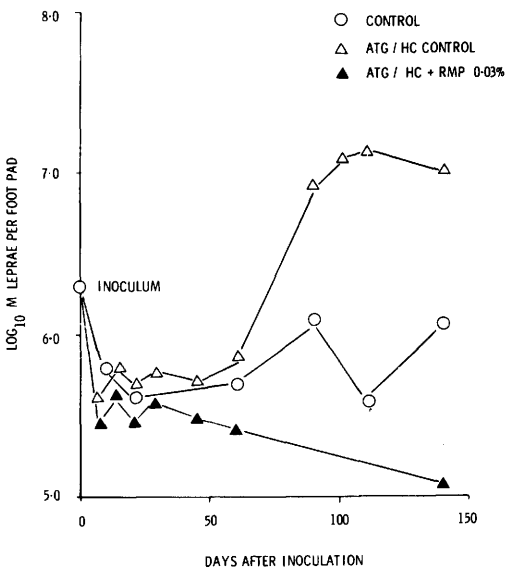

FIG. 4. Enhanced growth of a large inoculum (2.0×10^6) of *M. leprae* in footpads of mice immunosuppressed from the day of inoculation. Growth inhibition by continuous dietary rifampin administration is also indicated. Immunosuppression was by the following regimen: anti-mouse thymocyte globulin (0.2 ml sc twice weekly) and hydrocortisone acetate (4.0 mg/kg/day im). Values are the mean of three replicates.

treated control group increased exponentially (mean generation time of 16 days) to a plateau count of 1.4×10^7/footpad 140 days postinoculation (Fig. 4); total bacillary numbers in normal mice remained essentially unaltered, and in RMP-treated mice there was an exponential fall. Figure 5 illustrates the changes in S.R. in control, normal, and immunosuppressed mice and in RMP-treated immunosuppressed mice. In ATG/HC-control mice there was a steady increase in S.R. from 18% to a maximum of 23% 87 days after inoculation, showing that during the lag period of the total counts there was disappearance of nonsolid organisms balanced by growth of the viable part of the inoculum. The fluctuating course of the S.R. in control immunologically competent mice reflected the phasic character of the plateau stage of *M. leprae* growth in mice, previously reported by Shepard and McRae (19). In the absence of any effect of host immunity in the RMP-treated group, the S.R. of *M. leprae* decreased exponentially during the first 42 days of treatment with a $t_{1/2}$ of solid bacilli of 12–13 days.

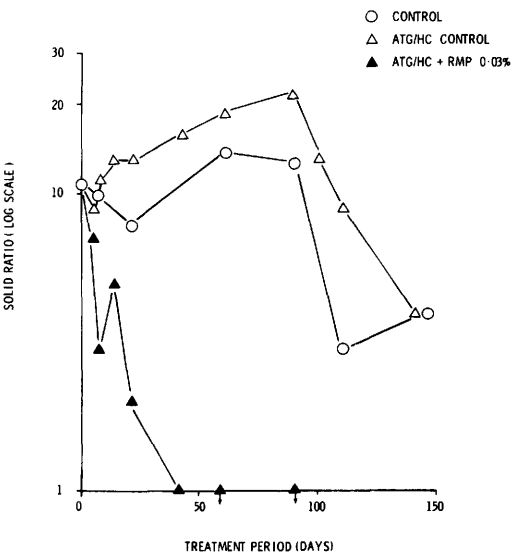

FIG. 5. The effect of continuous dietary administration of rifampin (RMP; 0.03%) on the solid ratio of *M. leprae* in immunosuppressed mice. The immunosuppressive regimen was as in Fig. 4. Values are the mean of three replicates. Arrow (↓) indicates a solid ratio of less than one.

Drug administration for 63 days was sufficient to reduce the S.R. to zero, at a time when the value for ATG/HC controls was 19%.

Discussion. Shepard and Chang (20) failed to detect any significant reduction by dapsone (DDS) of the S.R. of *M. leprae* in an established mouse footpad infection over and above the decrease due to host immunity. The more rapid bactericidal action of RMP (5, 21) allowed the detection of an additional effect of this drug (Fig. 1). The $t_{1/2}$ of solid bacilli in control animals exposed to the action of host immunity was 26 days; that in RMP-treated mice was 7 days. Thus, with allowance for the additional effect of immunity in treated animals, the $t_{1/2}$ of solid bacilli exposed to RMP was a little more than 1 week. This may represent a minimal estimate of RMP action since the exposed bacilli were already approaching the stationary growth phase. The fact that the total number and S.R. of *M. leprae* in control immunosuppressed mice increased steadily during the first 90 days of the experiment indicates that the fall in S.R. in RMP-treated immunosuppressed mice was entirely due to drug action (Fig. 4). The half-life of *M. leprae* exposed to RMP was in this case 12–13 days, a value which was double that for immunologically competent mice (6–7 days). In a previously reported study (5) the estimated survival half-life of *M. leprae* was 0.5 day (kinetic technique) and 0.6 day (subinoculation). Consequently, the estimations of $t_{1/2}$ values suggests that there is a delayed change in bacillary morphology (from solid to nonsolid) following loss of viability (mouse infectivity). Estimation of M.I. (mean solid ratio) is used as a basis for clinical assessment of drug bactericidal activity in leprosy. In the case of rapidly bactericidal drugs the delayed change in bacillary morphology would result in an underestimate of drug efficacy which would, however, be of minor clinical significance in view of the long-term nature of leprosy chemotherapy.

The rate of decrease of S.R. of *M. leprae* exposed to RMP in the present study is consistent with the findings in lepromatous leprosy patients treated with 600 mg/day of the drug (3); in them, the $t_{1/2}$ of the M.I. was 5 days. This dosage produces serum levels of 7–16 μg/ml (22, 23), similar to those in mice receiving 0.03% RMP in the diet (6–12 μg/ml) (24).

It is known from previous studies that the rate of bactericidal action of B663 and B1912 on *M. leprae*, as assessed by the kinetic technique involving mouse infectivity, is considerably slower than that of RMP (17). This was confirmed in the present study. Both drugs appear to permit a lag phase before exerting the bactericidal action. In view of this, and the subsequent small degree of enhancement of the effect of host immune response, it is reasonable to consider B663 and B1912 as only weakly bactericidal. The same conclusion holds for DDS: Its low degree of bactericidal action has been previously reported on several occasions (25). The present results with B663 correlate well with reported clinical observations based on estimations of M.I. Browne (14) reported that only after continuous treatment of lepromatous leprosy patients with B663, 300 mg/day for 150 days, was the M.I. reduced from 56 to 3%. A similar treatment period was necessary for a 90% reduction of M.I. in another study (15). It is thus clear that both in clinical and experimental chemotherapy, the reduction of S.R. due to B663 is much slower than that due to RMP. No clinical data are available in the case of B1912, but experimental findings demonstrated a low rate of bactericidal action, similar to that of B663 (17).

Comparison of serum levels of riminophenazines in mice with those in man are unhelpful. There is a poor correlation between drug dosage and serum levels, and intracellular accumulation of drug occurs. However, treatment of patients with B663 300 mg/day produces serum levels of 0.6 to 3.0 μg/ml (26) and treatment of mice with 0.01% dietary B663 produced a serum level of 2.4 μg/ml (29).

The progressive accumulation of the riminophenazines in tissues and their prolonged persistence after cessation of administration (27, 28, 29) has led several investigators to study the potential of B663 for chemoprophylaxis and intermittent therapy in experimental tuberculosis and leprosy in mice (30, 31). In this investigation, although the dose per kilogram per day of B663 and B1912 administered in the intermittent regimen

was greater than that when given continuously (75 mg versus 25 mg), treatment was for a total period of 8 days only. The total amount of drug administered was therefore one-tenth that given by continuous administration. The rates of bactericidal action, after the normal initial delay period were, however, very similar to those observed with continuous administration. Clinical observations of the effect of intermittent B663 therapy are conflicting (Goodwin, personal communication; 32) and more extensive trials may provide useful information of the usefulness of intermittent therapy with B663 in the treatment of leprosy.

Conclusions. In view of the close correlation between the experimental results presented and previously reported clinical findings with RMP and B663, it is felt that determination of the effect of drugs on the S.R. of *M. leprae* in the mouse footpad can provide a useful estimate of potential bactericidal activity in clinical leprosy infections.

Summary. Continuous dietary administration of rifampin to mice with an established *Mycobacterium leprae* footpad infection reduced the bacillary solid ratio, with an estimated survival half-life of 5–6 days. In rifampin-treated immunosuppressed animals the survival half-life of solid bacilli, in the absence of host immunity, was 12–13 days. Clofazimine and B1912 produced a significant effect on solid ratio only after a lag period of apparently 100 days. The rate of action was considerably slower than that of rifampin. Intermittent (once monthly) administration of both drugs produced effects similar to those of continuous administration.

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